REVIEW

# New Insights into Soybean Biological Nitrogen Fixation

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## ABSTRACT

Soybean biological $N_2$ fixation (BNF) relationships with fertilizer N and yield response have been comprehensively reviewed in the scientific literature. However, the study of the N-gap between N uptake and N supplied by $N_2$ fixation, and the partial N balance (fixed N in aboveground biomass – N seeds) needs further investigation. Therefore, the goals of this synthesis–analysis were to (i) quantify seed production per unit of fixed N under different amounts of N derived from the atmosphere (NDFA, %), (ii) study the N-gap and explore limitations of $N_2$ fixation (kg ha$^{-1}$) for satisfying plant N demand, and (iii) calculate a partial N balance for soybean and determine its relationship with the $N_2$ fixation process. Data was gathered from 1955 through 2016 using studies reporting BNF, seed yield, and plant N uptake ($n = 733$ data points). The main outcomes of this review were (i) as NDFA increased, seed production per $N_2$ fixation decreased (from 0.033 to 0.017 Mg yield kg$^{-1}$ N from low, 28%, to high, 80%, NDFA); (ii) N-gap increased faster when NDFA values were above 80% and after plant N content was above 370 kg N ha$^{-1}$ suggesting that the crop needs additional N for coping yield potential; and (iii) when excluding roots, the partial N balance calculation revealed negative values across all NDFA levels. Future studies should consider a holistic approach to quantify the contribution of BNF in overall N cycling, including N contribution from roots, and to better understand the soil × plant × rhizobia interactions.

## Core Ideas

- As $N_2$ fixation (%) increased, seed production per $N_2$ fixation decreased.
- The N-gap between crop N uptake and N supplied by $N_2$ fixation rose when contribution from biological $N_2$ fixation increased.
- The partial N balance revealed negative values across all N derived from the atmosphere levels.
- Yield was negatively related to partial N balance when N derived from the atmosphere was below 42%.

Soybean (*Glycine max* L.) is one of the most important crops grown worldwide as source of both protein and oil. Three countries are the main soybean producers at the global scale: Argentina, Brazil, and the United States; they comprise 16, 32, and 33%, respectively, of the estimated global soybean production (USDA NASS, 2017). From a historical perspective, soybean yield, harvested area, and total production have been increasing in all three countries but at different rates (SoyStat, 2016). Historical gains in soybean seed yields are primarily due to increases in seed biomass, which demonstrates an improvement in seed partitioning efficiency (Koester et al., 2014). The increase in partitioning efficiency and seed biomass requires larger N demand (Balboa et al., 2018), primarily met by biological $N_2$ fixation (BNF) and soil N mineralization.

In soybean, N derived from the atmosphere (NDFA) via BNF can range from 0 to 98% of the total N uptake, representing 0 to 337 kg N ha$^{-1}$ (Salvagiotti et al., 2008), depending on rhizobia activity. However, N removal from the system (i.e., by seed N) is determined by different factors that affect seed yield and N harvest index (NHI; seed N uptake to total N uptake). In a recent study, Tamagno et al. (2017) showed that NHI in soybean grown in three different regions ranged from 44 to 91% (at R7–R8 growth stages), with yields ranging from ~1 to 8 Mg ha$^{-1}$. The previous information related to NDFA contribution and NHI calculation for soybeans portrays the complexity of estimating N contribution of soybeans to the rotation. A review study summarizing 108 scientific papers published from 1966 to 2006 documented an average NDFA contribution of 50 to 60% (Salvagiotti et al., 2008). Comparable NDFA estimations were documented in Argentina: 60% (ranging 46–71%) (Collino et al., 2015) and up to 80% in less fertile soils in Brazil (Alves et al., 2003). The review by Salvagiotti et al. (2008) suggested that the NDFA contribution was not sufficient for high-yielding soybeans (>7 Mg ha$^{-1}$). Salvagiotti et al. (2009) showed a slight increase in seed yield in crops that yielded more than 5 Mg ha$^{-1}$ when N was supplied without affecting the $N_2$ fixation process. However, the question, whether $N_2$ fixation alone can supply N for a high-yielding soybean while maintaining a neutral partial N balance (fixed N in aboveground biomass minus N removed in seeds), remains unanswered.

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**Abbreviations:** BNF, biological $N_2$ fixation; NHI, N harvest index; NIE, N internal efficiency; N-seed, seed N content, NDFA, N derived from the atmosphere.

The "N-gap" between crop N uptake and N supplied by $N_2$ fixation has not yet been estimated at varying yield and NDFA levels. A better understanding of the so-called N-gap could allow development of potential N management strategies to further boost soybean yields and profitability. These strategies should take into account the trade off between inorganic N fertilizer and fixed N (Salvagiotti et al., 2008). High-yielding soybean environments should be accompanied by high $N_2$ fixation activity, as suggested by van Kessel and Hartley (2000). Collino et al. (2015) showed greater $N_2$ fixation as yield potential increased. However, if $N_2$ fixation contribution remains constant (or increasing less than proportional to yield) at increasing productivity levels, then mineral soil N contribution should meet the crop demand. Consequently, N could be mined from the soil producing negative partial N balance and affecting soil health.

Soybean breeding efforts have been focused on improving partitioning efficiency (seed yield per unit of biomass) (Kumudini et al., 2001, 2002; Koester et al., 2014) and increasing the duration of early reproductive stages. However, the reduction in $N_2$ fixation rates between R5 and R7 stages (Zapata et al., 1987), even when not always consistent, could lead to N limitation during seed filling, as recently documented by Tamagno and Ciampitti (2017). The same authors found that treatments with high fertilizer N rate extended the duration of the seed filling, without modifying seed growth rate, by 5 d as compared with treatments depending only on BNF, with overall yields for the high N fertilization of 4 Mg ha$^{-1}$. Thus, it can be hypothesized that reaching maximum yield cannot solely depend on $N_2$ fixation to satisfy soybean N demand. Following this rationale, a better assessment of the N-gap between crop N uptake and N supplied by $N_2$ fixation is needed.

In summary, three main critical points will be addressed in this review: (i) quantify seed production per kg of fixed N under different NDFA proportions, (ii) quantify N-gap and explore limitations of $N_2$ fixation for satisfying plant N demand, and (iii) calculate a partial N balance for soybean and determine its relationship with $N_2$ fixation and provide an overall value for the fixed N contribution to the "soybean N credit" or "soybean rotation effect", excluding the potential contribution of N from BNF coming from the roots.

## DATABASE AND ANALYSIS

### Database

The database consisted of information previously collected from scientific literature over the past decades (Salvagiotti et al., 2008: 38 studies), but it also included 22 additional scientific studies, for a total of 60 datasets from the entire globe from 1955 through 2016 (Table 1). In all cases, the database comprised studies primarily focused on quantifying $N_2$ fixation in soybean as affected by multiple genotypes × environment × management practices combinations. Countries represented in the database included the United States (15 datasets, $n$ = 201 observations), China (7, $n$ = 91), Thailand (6, $n$ = 92), Argentina (4, $n$ = 113), Australia (4, $n$ = 58), Brazil (4, $n$ = 17), Japan (4, $n$ = 28), Canada (2, $n$ = 41), Austria (3, $n$ = 22), India (2, $n$ = 13), Nigeria (2, $n$ = 15), France (1, $n$ = 8), Indonesia (1, $n$ = 2), Syria (1, $n$ = 9), Kenya (1, $n$ = 8), South Korea (1, $n$ = 3), Zambia (1, $n$ = 4), and Zimbabwe (1, $n$ = 8). Data inclusion followed criteria defined in previous review papers (Ciampitti and Vyn, 2012, 2013, 2014; Ciampitti and Prasad, 2016). Briefly, information must report data on seed yield at harvest and data at the end of the crop cycle (mainly R6.5–R7) of total plant N content, $N_2$ fixation (g m$^{-2}$), NDFA (%), and seed N content. Multiple $N_2$ fixation determination techniques have been reported in different studies: (i) N difference method (8 studies), (ii) ureides determination in the xylem sap or stems (17 studies), (iii) $^{15}$N dilution technique and $^{15}$N natural abundance technique (34 studies), and (iv) acetylene reduction method (1 study); for complete citations of all techniques refer to Salvagiotti et al. (2008) and Unkovich and Pate (2000). The majority of data was retrieved from tables, some from equations, and a small proportion from digitized figures; if only NDFA was reported, total magnitude of N fixed was determined by multiplying the NDFA (%) contribution by its respective plant N content per unit area value. When BNF was estimated by the ureide or the acetylene reduction method, $N_2$ fixation was calculated by integrating measurements that were made multiple times over the season (i.e., there were not point-in-time measurements) (Peoples et al., 1989).

### Variables Evaluated

For the purpose of this review, total plant N content refers to the aboveground plant N content (stem, leaves, podwalls, and seed) determined close to maturity (as describe above, R6.5–R7), excluding belowground fraction. A similar concept was followed for the BNF variables, excluding any contribution from roots, because most of the studies did not provide this information. If not reported in the original publication, plant N content per unit area was determined by multiplying plant biomass and its respective N concentration (dry mass basis). Total $N_2$ fixation was reported in the research studies or calculated as the $N_2$ fixation (%) multiplied by its respective plant N content. For this review, the term $N_2$ fixation was utilized to refer the quantity of soybean N fixed at the end of the season (kg N ha$^{-1}$), while the term NDFA (%) refer to the contribution of $N_2$ fixation as a proportion of plant N content, expressed in relative terms. Seed yield was reported on an area basis and adjusted to 13% moisture content.

The partial N balance was calculated as follows:

Partial N balance = Fixed N in
aboveground biomass – N seeds.

Negative partial N balance indicates that the amount of N exported from harvesting soybean seeds is larger than N fixed by the crop, and thus a net "soil N depletion" may occur. Alternatively, a positive partial N balance portrays a situation where $N_2$ fixation contribution to the soybean exceeds seed N export, resulting in a net positive N budget. To consider the potential contribution of roots, an adjusted partial N balance was calculated by assuming that 24% of the total plant N content is located in the roots (Rochester et al., 1998).

### Database Analysis

For the entire database, a descriptive analysis was performed using the R function "hist" to prepare all histograms (R Development Core Team, 2009) and to estimate total number

Table 1. Number of study, country, experimental design, year of experimentation, BNF method, fertilizer N rates, and treatments evaluated for each different soybean study included in the meta-database. Other studies from Salvagiotti et al. (2008)†.

| No | Country | Author, Yr | Design | Years | BNF Method | N rate | Treatments evaluated |
|---|---|---|---|---|---|---|---|
| 1 | China | Yong et al., 2015 | Split-plot | 2012/13 | $^{15}$N natural abundance | 3 (0, 180, 240 kg N ha$^{-1}$) | Planting patterns × N rates × crop rotation |
| 2 | Syria | Al-Chammaa et al., 2014 | Randomized Complete Block (RCB) | Not reported | $^{15}$N isotopic dilution | 20 kg N ha$^{-1}$ as labeled | Phosphorus levels × rates of sheep manure |
| 3 | Japan | Tewari et al., 2005 | Not reported | 2002 | $^{15}$N isotopic dilution | 16 basal + 100 kg N ha$^{-1}$ | Deep placement of $^{15}$N-labeled × inoculation |
| 4 | Argentina | Di Ciocco et al., 2008 | RCB | 2004/05 | $^{15}$N using three methods | 10 kg N ha$^{-1}$ as labeled ammonium sulfate | BNF methods × tillage system |
| 5 | Nigeria | Sanginga et al., 1997 | Split-/Split-split-plot | 1994/95 | $^{15}$N isotopic dilution | 20 kg N ha$^{-1}$ as labeled | Varieties × growth periods × inoculation |
| 6 | Nigeria | Sanginga et al., 2002 | RCB | 1996/97 | $^{15}$N isotopic dilution | 20 kg N ha$^{-1}$ as labeled | $^{15}$N labeling approach × varieties |
| 7 | Kenya | Kihara et al., 2011 | Factorial | 2007 | $^{15}$N isotopic dilution | 4,6 kg N ha$^{-1}$ as labeled | Tillage × crop residue × cropping systems |
| 8 | India | Singh et al., 2014 | RCB | 1972/10 | N balance method | 10 different N rates | Nutrient management in soybean and wheat |
| 9 | South Korea | Park et al., 2005 | RCB | 2001/02 | Ureides | 0 or 3 kg N ha$^{-1}$ | Cropping systems × N levels × varieties |
| 10 | Japan | Shimada et al., 2012 | Split-plot | 2006/07 | Ureides | 36 kg N ha$^{-1}$ as dolomitic limestone | Water table × soybean varieties |
| 11 | USA | Salvagiotti et al., 2009 | RCB | 2006/07 | Ureides | 0 or 180 kg N ha$^{-1}$ of different fertilizer sources | N fertilizer management |
| 12 | Zimbabwe | Zingore et al., 2008 | RCB | 2002/05 | $^{15}$N natural abundance | 70 kg N ha$^{-1}$ as ammonium nitrate (maize) | Crop rotation × manure |
| 13 | Brazil | Alves et al., 2006 | RCB | 2000/02 | $^{15}$N natural abundance | No–N reported | Crop rotation |
| 14 | Brazil | Macedo and Miranda, 2001 | RCB | 1997/98 | $^{15}$N natural abundance | No–N reported | Tillage systems × crop rotation |
| 15 | Brazil | Neves et al., 1985 | RCB | 1983 | Ureides | 2 (0 or 60 kg N ha$^{-1}$) | N rates × inoculation |
| 16 | Argentina | Collino et al., 2015 | On-farm research | 2004/11 | $^{15}$N natural abundance | No–N reported | On–farm research |
| 17 | Canada | Lynch and Smith, 1993 | Completely Randomized | Not reported | N difference | No–N reported | Exposure to a low root–zone temperature |
| 18 | China | Xinmin et al., 1993 | RCB | Not reported | $^{15}$N isotopic dilution | No–N reported | Varieties and ability to fix N |
| 19 | China | Mengpei et al., 1986 | Not reported | 1982–1985 | $^{15}$N isotopic dilution | N check, and N–P–K combinations | Fertilizer rates × residue on yields and BNF |
| 20 | China | Jinfan et al., 1987 | Not reported | 1985 | $^{15}$N isotopic dilution | No–N reported | N fixation on three soils × soybean varieties |
| 21 | Argentina | Santachiara et al., 2017 | RCB | 2012/13 | $^{15}$N natural abundance | No–N reported | Soybean varieties (70 genotypes) × N fixation |
| 22 | USA | Tamagno and Ciampitti, 2017 | RCB | 2016 | Ureides | 0 or 112 kg N ha$^{-1}$ | N rates × timing × N fixation across several sites in the US Midwest |

† From Salvagiotti et al. 2008 (38 studies): Alvarez et al., 1995; Gan et al., 2002; Hughes and Herridge, 1989; Zotarelli, 2000; Bezdicek et al., 1978; Weber, 1966; Kucey et al., 1988a,b; Rennie et al., 1988; Amarger et al., 1979; Zapata et al., 1987; George et al., 1988; Kundu et al., 1996; Sisworo et al., 1990; Afza et al., 1987; Hardarson et al., 1984; Jefing et al., 1992; Guafa et al., 1993; Guffy et al., 1989; Peoples et al., 1995; Bergersen et al., 1992; Cassman et al., 1993; Leffel et al., 1992; Vasilas and Ham, 1985; Munyinda et al., 1988; Ham and Cadwell, 1978; Bhangoo and Albritton, 1976; Johnson et al., 1975; Ravuri and Hume, 1993; Vasilas and Fuhrman, 1993; Herridge, 1982; Zhang et al., 1986; Thies et al., 1995; Toomsan et al., 1995; Takahashi et al., 1991; Gan et al., 2002; Tewari et al., 2004; Israel and Burton, 1997.

Table 2. Descriptive statistics of the meta-database relative to soybean yield (adjusted to 13% moisture), Plant N content at the end of the season (dry basis), N contribution from biological N fixation ($N_2$ fixation) at the end of the season in absolute values and expressed in relative terms, N derived from the atmosphere, NDFA %, in aboveground biomass.

| Parameter | Unit | n | Mean | SD | Minimum | 25% Q | Median | 75% Q | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| Seed yield | Mg ha$^{-1}$ | 733 | 3.1 | 1.4 | 0.10 | 2.0 | 2.9 | 4.1 | 8.3 |
| Plant N content | kg ha$^{-1}$ | 733 | 245 | 108 | 7.0 | 162 | 228 | 331 | 538 |
| $N_2$ fixation (all N) | kg ha$^{-1}$ | 733 | 137 | 82 | 0.0 | 72 | 127 | 194 | 372 |
| $N_2$ fixation (no N) | kg ha$^{-1}$ | 473 | 142 | 78 | 0.0 | 83 | 130 | 194 | 372 |
| NDFA % (all N†) | Unitless | 733 | 56 | 21 | 0.0 | 44 | 59 | 72 | 98 |
| NDFA % (no N‡) | Unitless | 473 | 58 | 19 | 0.0 | 46 | 60 | 73 | 98 |

† All N refers to summary statistics for all data sets, including treatments with or without fertilizer-N.

‡ No N refers to summary statistics for the data sets without including any fertilizer-N.

Table 3. Descriptive statistics of the meta-database relative to the partial N balance, calculated as the N contribution $N_2$ fixation minus the total N removed in the seed, seed yield (adjusted to 13% moisture), seed N content (dry basis), $N_2$ fixation (absolute terms) at the end of the season (mainly R6.5 to R7 growth stage), N derived from the atmosphere, and NDFA (expressed in relative terms to total plant N content), for each NDFA group (as defined in Fig. 1A).

| Parameter | Unit | *n* | Mean | SD | Minimum | 25% Q | Median | 75% Q | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| Partial N balance (all N†) | kg ha$^{-1}$ | 460 | –47 | 55 | –279 | –76 | –38 | –11 | 111 |
| Partial N balance $_{roots}$‡ | kg ha$^{-1}$ | 460 | –13 | 66 | –279 | –46 | –4.8 | 29 | 181 |
| Seed yield | Mg ha$^{-1}$ | 460 | 3.2 | 1.5 | 0.1 | 2.0 | 3.0 | 4.3 | 8.3 |
| Seed N content | kg N ha$^{-1}$ | 460 | 183 | 84 | 3.9 | 125 | 167 | 251 | 409 |
| $N_2$ fixation | kg ha$^{-1}$ | 460 | 136 | 86 | 0.0 | 66 | 127 | 198 | 372 |
| NDFA$_{\%}$ | Unitless | 460 | 55 | 21 | 0.0 | 43 | 58 | 71 | 94 |
| Partial N balance (no N§) | kg ha$^{-1}$ | 190 | –33 | 49 | –159 | –60 | –35 | –2 | 110 |
| NDFA$_{\%}$ Groups | | | | | | | | | |
| | | | | | 0–44% NDFA | | | | |
| Seed yield | Mg ha$^{-1}$ | 122 | 2.9 | 1.2 | 0.5 | 1.8 | 2.8 | 3.4 | 6.1 |
| Partial N balance | kg ha$^{-1}$ | 122 | –100 | 55.1 | –279 | –141 | –106 | –51.0 | 5.0 |
| $N_2$ fixation | kg ha$^{-1}$ | 122 | 62.5 | 49.2 | 0.0 | 25.4 | 50.5 | 89.0 | 197 |
| | | | | | 44–72% NDFA | | | | |
| Seed yield | Mg ha$^{-1}$ | 236 | 3.2 | 1.5 | 0.1 | 2.1 | 3.0 | 4.4 | 7.3 |
| Partial N balance | kg ha$^{-1}$ | 236 | –38.5 | 38.7 | –153 | –59.0 | –37.0 | –14.0 | 74.0 |
| $N_2$ fixation | kg ha$^{-1}$ | 236 | 145 | 71.2 | 0.0 | 89.1 | 134 | 205 | 321 |
| | | | | | 72–98% NDFA | | | | |
| Seed yield | Mg ha$^{-1}$ | 102 | 3.6 | 1.7 | 0.4 | 2.4 | 3.3 | 4.7 | 8.3 |
| Partial N balance | kg ha$^{-1}$ | 102 | –3.4 | 33.0 | –80.0 | –26.2 | –5.0 | 8.2 | 110.0 |
| $N_2$ fixation | kg ha$^{-1}$ | 102 | 202 | 86 | 26.0 | 135 | 198 | 268 | 372 |

† All N refers to summary statistics for the partial N balance, including treatments with or without fertilizer-N.

‡ Partial N balance $_{roots}$, calculations assuming an average N contribution from belowground biomass of 24% (Rochester et al., 1998).

§No N refers to summary statistics for the partial N balance without including any fertilizer-N.

of observations (*n*), mean, standard deviation (SD), minimum, 25–75% quartile, median, and maximum all variables collected in this paper (Tables 2 and 3). Two databases were formed with equal numbers of observations for (i) quantify N-gap including seed yield (Mg ha$^{-1}$), plant N content (kg N ha$^{-1}$), $N_2$ fixation (kg N ha$^{-1}$), and NDFA (%) (Database 1, Table 2); and (ii) estimate partial N balance comprising seed yield and $N_2$ fixation (Database 2, Table 3). Database 1 is presented in Fig. 1, 2, and 3 (Table 2), while Database 2 was utilized for Fig. 4 (Table 3). Histograms were calculated for NDFA, seed yield, and plant N content (Fig. 1A, B, C), with Gaussian models fitted for each NDFA group (GraphPad Prism 6; Motulsky and Christopoulos, 2003). For the frequency distributions of NDFA, three groups were formed from < 25th, 25th–75th, and >75th: (i) low NDFA 0–44% (*n* = 187); (ii) medium NDFA 44–72% (*n* = 372); and (iii) high NDFA 72–98% (*n* = 174) (Fig. 1A). The seed yield-to-plant N content (Fig. 2A) relationship was characterized by determining envelopes portraying the maximum and minimum boundaries, 0.99 and 0.01 quantiles (Koenker, 2005). The linear components of the relationship between seed yield and $N_2$ fixation were tested for each NDFA group (Fig. 2B) (F test, Mead et al., 1993) and compared with a global fit (GraphPad Prism 6, Motulsky and Christopoulos, 2003). These relationships were also shown by method of estimating $N_2$ fixation to check for potential literature bias in this review (Supplementary Fig. S1). There was no clear trend of BNF method as related to low or high yields, plant N content, and/or $N_2$ fixation values. Quantile regression was also utilized to estimate (Koenker, 2005) 10, 25, 50, 75, and 99 percentiles, for the relationship between $N_2$ fixation and plant N content (Fig. 3A). In addition, a boundary function (0.99 quantile) was fitted to identify the expected highest fixed N values for a given level of plant N content. Linear and quadratic models were tested for each quantile regression and tested for equality of slopes among percentile lines. A similar approach was previously implemented by several researchers for identifying the highest yields for a given resource supply

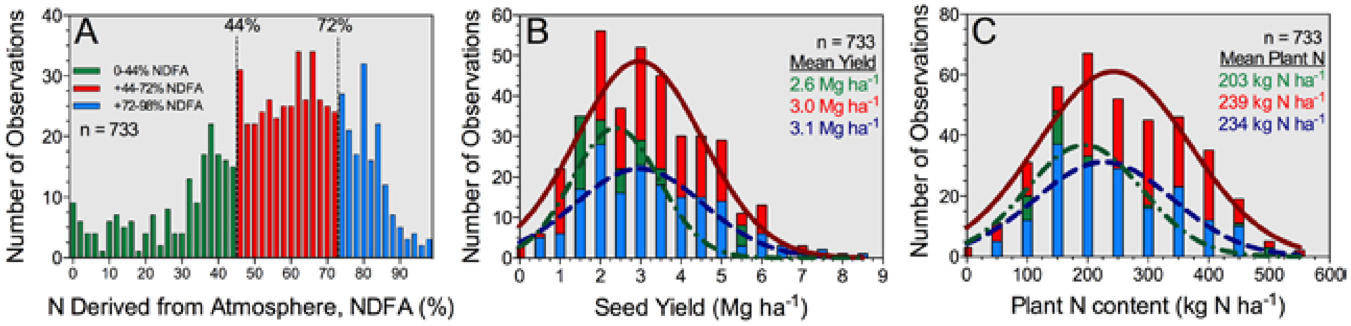

Fig. 1. Histogram of frequency for contribution of N from biological N fixation (BNF), expressed as the N derived from the atmosphere (NDFA) (A), seed yield, expressed in Mg ha$^{-1}$ and adjusted to 13% moisture content (B), and plant N content, expressed in kg N ha$^{-1}$ in dry basis (C), all relative to the NDFA groups: 0 to 44% NDFA (green); +44 to 72% NDFA (red), and +72 to 98% NDFA (blue). Lines in (B) and (C) represent the Gaussian fit for each BNF group.

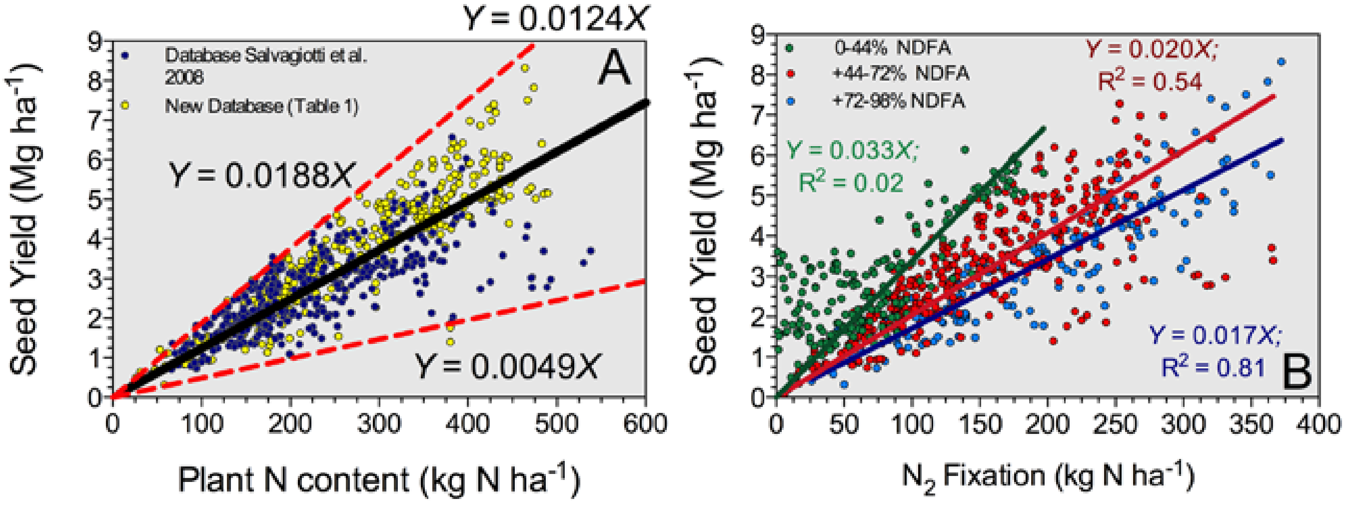

Fig. 2. Relationship between seed yield (adjusted to 13% moisture content) versus plant N content (dry basis) (A), and $N_2$ fixation (kg N ha$^{-1}$) for different NDFA groups (B). In (A), the solid line is the average fit of the data, with a slope of 0.0124 Mg grain kg$^{-1}$ N. Dashed red lines show the boundaries of maximum N dilution (upper) and maximum N accumulation (lower). In (B), green, red, and blue lines depict the best-fitted line for the 0 to 44%, +44 to 72% and +72 to 98% NDFA groups, respectively.

(Tittonell et al., 2008; Hochman et al., 2009). Changes in the $N_2$ fixation to plant N content ratio for the boundary function were further studied via calculation of a segmental linear regression for values of plant N content above 200 kg N ha$^{-1}$ and then 370 kg N ha$^{-1}$. The N-gap, calculated as the difference between crop N uptake and N supplied by $N_2$ fixation for each quantile regression line was plotted against plant N content to quantify changes in response models for this relationship (Fig. 3B). The partial N balance was studied for understanding its statistical distribution (Fig. 4A), cumulative frequency (Fig. 4B), and final association with seed yield (Fig. 4C) as related to the three groups previously defined for NDFA (Fig. 1A).

## SEED YIELD, PLANT N CONTENT, AND $N_2$ FIXATION

The analysis of the pooled data for yield and plant N content indicated a similar variation considering the 50% interquartile range (50% IQR, from 25–75% quartile), from 2.0 to 4.1 Mg ha$^{-1}$ for seed yield and from 162 to 331 kg N ha$^{-1}$ for plant N content (Table 2). Overall mean seed yield was 3.1 Mg ha$^{-1}$ with yield distribution slightly skewed toward high values (Fig. 1B) and a maximum value of 8.3 Mg ha$^{-1}$ (Table 2). Slightly lower mean seed yield and maximum value for yield were documented by Salvagiotti et al. (2008), which were 2.7 Mg ha$^{-1}$ and 5.9 Mg ha$^{-1}$, respectively. Therefore, this review increased the number of cases with high yields (Table 2). Plant N content at maturity averaged 245 kg N ha$^{-1}$, which also was slightly greater than the 219 kg N ha$^{-1}$ mean value documented by Salvagiotti et al. (2008).

Mean fixed N was 137 kg N ha$^{-1}$, showing a maximum value of 372 kg N ha$^{-1}$ (Table 2). In relative terms, $N_2$ fixation (%) reached the maximum point at 98%. Overall, the NDFA was 56% and presented a 50% IQR from 44 to 72% (Table 2). Under dryland soybean conditions, NDFA was reported to be 50% (Unkovich and Pate, 2000). In a large study from 41 sites in Argentina, average NDFA was 58%, with a 50% IQR from 46 to 71% (Collino et al., 2015). An overall NDFA of 52% was synthesized by Salvagiotti et al. (2008), with a 50% IQR ranging from 36 to 69%. When the database did not comprise studies that applied fertilizer-N, fixed N increased to a mean value of 142 kg N ha$^{-1}$ (from 137 kg N ha$^{-1}$) and an overall NDFA of 58% (from 56%) (Table 2). Thus, NDFA for the BNF process could range from 50 to 60% for soybean systems around the globe.

The N internal efficiency (NIE, i.e., the slope of the yield-to-plant N content relationship) was 0.0124 Mg kg$^{-1}$ N (Fig. 2A). The new dataset provided in this review (yellow circles; Fig. 2A) added higher yields and associated plant N content levels as compared to Salvagiotti et al. (2008). Superior yield values required

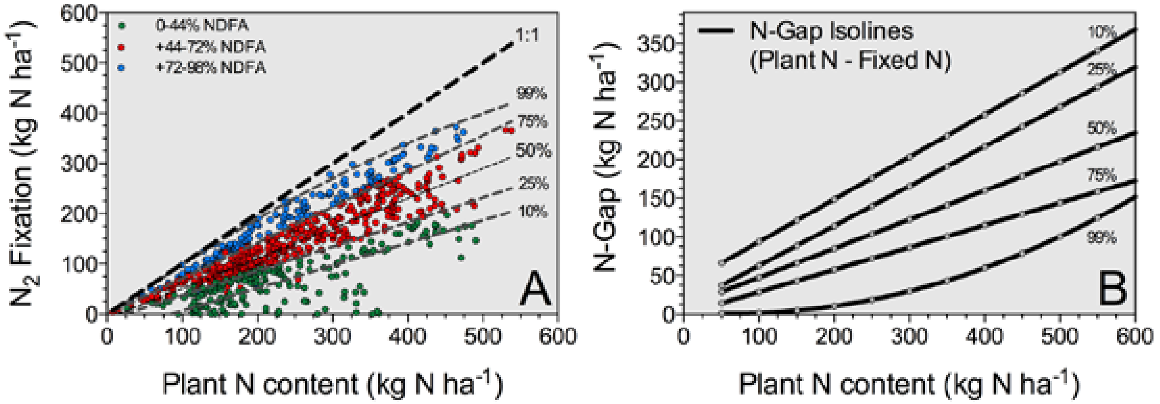

Fig. 3. Relationship between the contribution of $N_2$ fixation and plant N content (both expressed in kg N ha$^{-1}$) (A), and the N-gap (plant N– fixed N) relative to the plant N content (B). Quantile regression lines were fitted for the relationship in (A), representing NDFA isolines. For (A), the percentile 99 line (boundary function) adjusted was $Y = -3.47 + 1.07X - 0.0005X^2$. For (B), quantile regression lines, in (A), were used to calculate isolines of the difference between plant N content minus fixed N, herein term as N-gap isolines.

greater plant N content, but variation in this factor increased with yields; note the maximum dilution (0.0188 Mg kg$^{-1}$ N) and accumulation (0.0049 Mg kg$^{-1}$ N) boundary lines. Similar NIE and boundaries for the yield-to-N content relationship were previously documented by Salvagiotti et al. (2008). Then, for a constant 300 kg N ha$^{-1}$, seed yield is expected to be 1.5, 3.6, and 5.6 Mg ha$^{-1}$ for the maximum N accumulation (minimum boundary), median (average), and N dilution (maximum boundary) curves, respectively (Fig. 2A). The latter portrays the differential internal N use efficiency in soybean and the influence of other factors namely environment, management, and cultivar. It seems from the distribution of data points in Fig. 2A that there is not a clear plateauing in seed yield at high levels of N uptake as was observed in other graminaceous crops like corn (*Zea mays* L.) (Setiyono et al., 2010), rice (*Oryza sativa* L.) (Witt et al., 1999), or wheat (*Triticum aestivum* L.) (Liu et al., 2006), where NIE decreases as N content increases, suggesting that, in soybean, NIE remains constant even at high levels of seed yield (above 7 Mg ha$^{-1}$). This different behavior between cereals and soybean (and maybe other legumes) might be related to the fact that total plant N content in the former will be limited by soil plus fertilizer N sources, while in the latter the BNF process is mostly satisfying many times when plant N demand is not provided by soil. In addition, larger N concentration in vegetative and reproductive organs in legumes (e.g., soybeans) relative to cereals (e.g., corn) (Tamagno et al., 2017; Ciampitti and Vyn, 2011) could be another source for explaining the differences in NIE. When compared with other legumes (pigeon pea, *Cajanus cajan L.*; and peanut, *Arachis hypogaea L.*), soybeans presented larger losses of N in leaves (finishing with lower N concentrations), thus presenting differential N dynamics (Devries et al., 1989). In summary, more data beyond 500 kg N ha$^{-1}$ are needed to confirm if NIE will remain in a constant trend for soybeans.

Seed yield and $N_2$ fixation were linearly related in each of the NDFA groups (Fig. 2B). Overall, the yield-to-$N_2$ fixation relationship presented a larger variability relative to the yield-to-N content association. For the low NDFA group (green circles; Fig. 2B), $N_2$ fixation presented a maximum value of 197 kg N ha$^{-1}$ and seed yield of 6.1 Mg ha$^{-1}$. In this group, when $N_2$ fixation was below 50 kg N ha$^{-1}$ and seed yield ranging from >0 to <4 Mg ha$^{-1}$, lower yield was obtained in parallel with low NDFA below 20% (Fig. 2B). This group was characterized by studies that included fertilizer N application that averaged 106 kg N ha$^{-1}$ across all treatments, with high nitrate values around the nodulation zone or presence of low soil pH values, ineffective rhizobia, or biotic stresses affecting the nodulation process (Salvagiotti et al., 2008). This group showed low NDFA and N content more dependent on soil (or fertilizer) N. Changes in the slope of the yield-to-$N_2$ fixation relationship were primarily explained by NDFA, with higher slope (0.020X or 50 kg N Mg$^{-1}$) for the medium group relative to the high NDFA group (slope 0.017X or 59 kg N Mg$^{-1}$) (Fig. 2B). The reduction of more than 50% on the yield-to-$N_2$ fixation slope from the low to the high NDFA group reflects a decrease in the degree of dependency to other external N sources (soil + fertilizer; Fig. 2B) for satisfying N demand in the latter group. When comparing the effects of N fertilization in this meta-analysis (i.e., grouping 0 kg N ha$^{-1}$ vs. >10 kg N ha$^{-1}$) (data not shown), the slope for the yield-to-$N_2$ fixation relationship was not statistically different, between the N-fertilized and the zero-N fertilization group, averaging 0.0192X (i.e., 52 kg N Mg$^{-1}$).

When fertilizer N was applied at rates above 100 kg N ha$^{-1}$, this slope was slightly modified, lowering $N_2$ fixation per unit of N uptake when considering each sub-database (slope = 42 kg N Mg$^{-1}$; $n = 259$, N fertilizer rate 110 kg N ha$^{-1}$, seed yield 3.0 Mg ha$^{-1}$, and $N_2$ fixation 129 kg N ha$^{-1}$; vs. slope = 48 kg N Mg$^{-1}$; $n = 292$, N fertilization 0 kg N ha$^{-1}$, seed yield 2.8 Mg ha$^{-1}$, and $N_2$ fixation 132 kg N ha$^{-1}$). These results suggest that application of N fertilizer partially inhibited $N_2$ fixation decreasing the efficiency of N fixed per unit of yield, but further testing at multiple sites should confirm this research outcome. Several estimations of "energy costs" comparing soil N uptake and N fixed biologically at the cellular level are elusive for answering the question whether BNF represents a significant cost for the plant (Schubert, 1982; Salsac et al., 1984; Andrews et al., 2009). Generally, several assumptions are made for this type of analysis, and different values may come up depending on processes taken into account in the calculations. Schubert (1982) estimated that the total cost for both, BNF and inorganic N uptake are 92 ATP. However, Andrews et al. (2009) estimated that energy costs for BNF is 5 to 7% greater than for nitrate plus ammonium uptake.

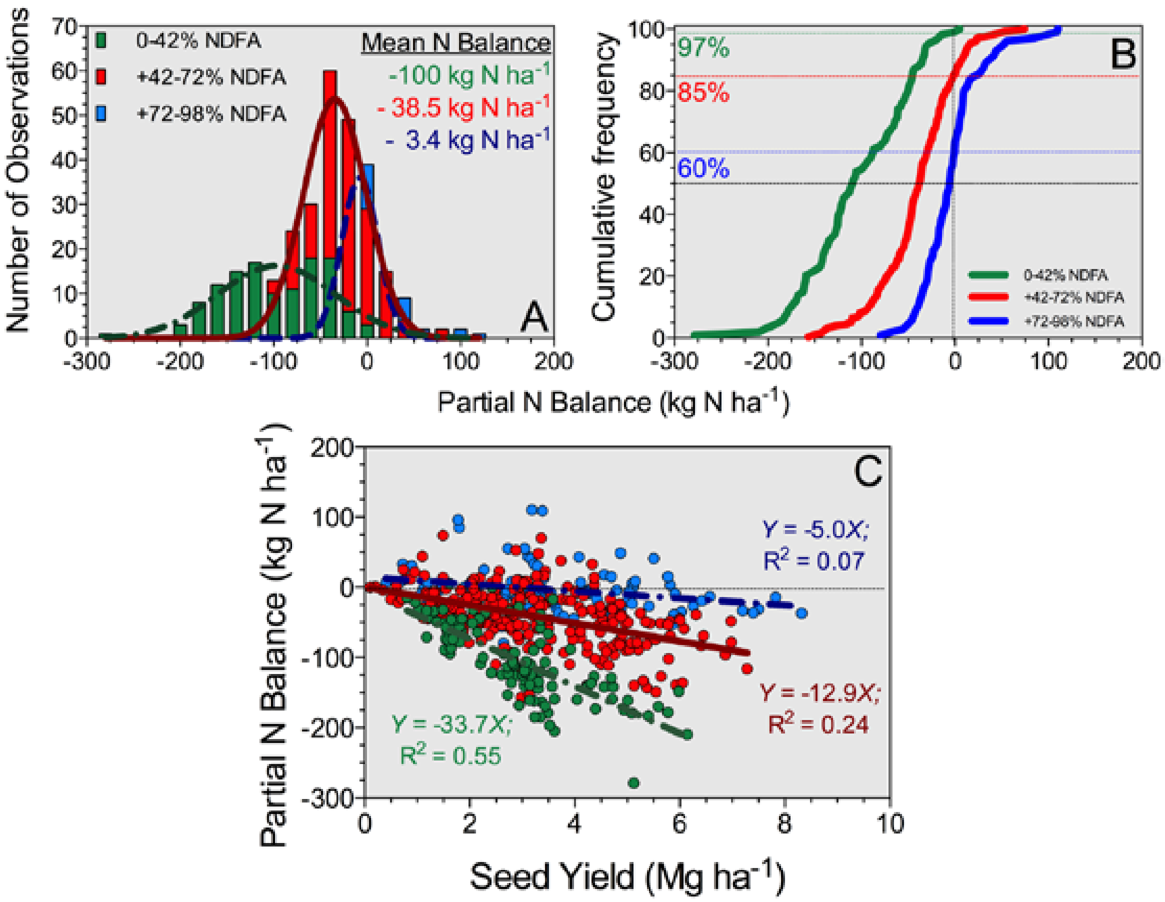

Fig. 4. Histogram of frequency for partial N balance (A), cumulative distribution frequency for partial N balance (B), and relationship between partial N balance and seed yield adjusted to 13% moisture content (C). Green, red, and blue lines depict the best-fitted line for the 0–44%, +44–72%, and +72–98% NDFA groups, respectively. Lines in (A) represent the Gaussian fit for each NDFA group.

## N-GAP AND MAXIMUM $N_2$ FIXATION

A relationship between $N_2$ fixation and plant N content was established to provide a better assessment of the N-gap that is plant N content minus $N_2$ fixation (Fig. 3A). Linear models better explained the relationship for percentiles 10 to 80 (percentile 80 not shown in the figure), but they presented different slopes among them. Regression lines from percentiles 10 to 25 shared an equal slope of 0.47X, but slopes increased less than proportionally until percentile 80 (i.e., percentile 50 and 75 showed a slope of 0.62X and 0.71X, respectively). Above percentile 80, a quadratic model better fit the relationship without changing the linear term of the equation between 90 and 99 percentiles (0.80X linear term) (Fig. 3A). Analyzing percentile 25 (low NDFA levels), when plant N content was 50 kg N ha$^{-1}$, the expected N-gap was 37 kg N ha$^{-1}$ and it rose to 183 kg N ha$^{-1}$ when plant N content was 400 kg N ha$^{-1}$ (i.e., an eight-fold increase in plant N demand was accompanied by a five-fold increase in the N-gap). However, when the NDFA increased (i.e., 75 quantile regression line), the N-gap was reduced close to 15 kg N ha$^{-1}$ when plant N content was 50 kg N ha$^{-1}$ and rose to 115 kg N ha$^{-1}$ when plant N uptake was 400 kg N ha$^{-1}$ (i.e., an eight-fold increase for both N content and N-gap) (Fig. 3B).

Many factors affect the $N_2$ fixation, including genotype × environment × management practices interactions. The main issue to be addressed in this review is to quantify the $N_2$ fixation maximum capacity. The boundary function (i.e., percentile 99 in Fig. 3A) represents the maximum attainable $N_2$ fixation contribution at each level of plant N content. As a first step the slope for the percentile 99 function was compared to the 1:1 line, and presented equal slope until 200 kg N ha$^{-1}$ (F test; Mead et al., 1993). The slope of −0.001 kg N fixed kg$^{-1}$ N uptake portrays that as plant N content increases N2 fixation decreases. As a second step, a segmental lineal regression was fitted for all plant N content values above 200 kg N ha$^{-1}$ to calculate the inflection point in which the N fixed to N uptake ratio changes, this plant N content value was obtained at 370 kg N ha$^{-1}$. Therefore, for the N-gap analysis, after 200 kg N ha$^{-1}$, the size of the N-gap increased at an estimated linear rate of 0.22 kg N-gap kg$^{-1}$ N content, and then drastically changing at 370 kg N ha$^{-1}$ with a slope of 0.46 kg N-gap kg$^{-1}$ N content, more than a twofold change (Fig. 3B). Thus, larger plant N content implies a greater dependency on external N sources to achieve higher yields. Maximum $N_2$ fixation values ranging from 337 to 372 kg N ha$^{-1}$ (with a concomitant NDFA from 68% to 86%) were gathered from Herridge (1982), Tewari et al. (2004), Santachiara et al. (2017), and Tamagno and Ciampitti (2017). In our dataset, only 3% ($n = 23$) showed seed yields above 6 Mg ha$^{-1}$, with an average NDFA of 67% (ranging from 33% to 81%), representing a mean $N_2$ fixation contribution of 279 kg N ha$^{-1}$ and with an N-gap of 137 kg N ha$^{-1}$. These results clearly show that soybean crops with a high contribution from BNF cannot attain high yielding (>7 Mg ha$^{-1}$) and potential N uptake. Very few observations ($n = 6$) attained yield levels above 7.5 Mg ha$^{-1}$ with an NDFA of 74%, total $N_2$ fixation contribution of 331 kg N ha$^{-1}$ and a N-gap of 116 kg N ha$^{-1}$. The latter outcomes are the first ones to concomitantly portray high-yielding soybean within the high NDFA group (+72%). In summary, N-gap of soybean that received a high proportion of N from BNF increased at a faster rate when overpassing 370 kg N ha$^{-1}$ (Fig. 3B), with overall mean and maximum yield environments from 4.6 to +7 Mg ha$^{-1}$ (Fig. 2A), respectively.

## PARTIAL N BALANCE AND $N_2$ FIXATION

The partial N balance (excluding BNF contribution from roots) presented an overall mean of −47 kg N ha$^{-1}$, with −75 to −11 kg N ha$^{-1}$ values for the 50% IQR (Table 3). For the dataset collected, only 17% of all observations showed a positive partial N balance, averaging 22 kg N ha$^{-1}$, while more than 80% of the data showed N balance of −61 kg N ha$^{-1}$ (Fig. 4A). Based on the NDFA groups (Fig. 1A), the partial N balance for the low BNF group presented an average of −100 kg N ha$^{-1}$ with a 50% IQR from −141 to −51 kg N ha$^{-1}$, an overall yield of 2.9 Mg ha$^{-1}$, and $N_2$ fixation of 62.5 kg N ha$^{-1}$ (Table 3). The medium NDFA group presented an average partial N balance of −38.5 kg N ha$^{-1}$ with a 50% IQR from −59 to −14 kg N ha$^{-1}$, a yield of 3.2 Mg ha$^{-1}$, and $N_2$ fixation of 145 kg N ha$^{-1}$. The high NDFA group presented an average of −3.4 kg N ha$^{-1}$ with a 50% IQR from −26 to 8 kg N ha$^{-1}$, with an overall yield of 3.6 Mg ha$^{-1}$, and $N_2$ fixation of 202 kg N ha$^{-1}$ (Table 3). The partial N balance for the high NDFA group presented a distribution centered around zero but with heavy tails toward both negative and positive values (Fig. 4A).

Cumulative frequencies for the partial N balance for each NDFA group are presented in Fig. 4B. For the low NDFA only 3% of the data ($n = 4$) presented positive partial N balance with 97% portraying a negative balance. The proportion of observations with positive partial N balance increased in the medium NDFA group, reaching 15% of all the datasets ($n = 35$). Lastly, the high NDFA group presented 40% of all observations with positive partial N balance ($n = 41$). Potential sources of error for the partial N balance are lack of accounting for potential N loss via leaf drop and the contribution of belowground parts. In the former case, N harvest index (used as an indicator of differences in N leaf drop) of the dataset evaluated was 0.70 units, ranging the 50% IQR from 0.60 to 0.80. Similar variation was recently documented by Tamagno et al. (2017) in a synthesis-analysis with an overall mean of 0.75 units. Then, this slight lower NHI difference obtained from two independent data sets allowed us to estimate N balances using the current data set. Nonetheless, N from dropped leaves is a potential source of error that should be properly estimated in future N balance studies for the soybean crop. Regarding the second source of error, i.e., N contribution from belowground parts, Table 3 shows a new balance including an additional 24% of N that is contributed by roots (Rochester et al., 1998), which still resulted in a −13 kg N ha$^{-1}$ balance (Table 3). Notwithstanding the adjustment of the partial N balance summing up this additional N contribution from BNF at R7 (Rochester et al., 1998), this method may still underestimate the total root N contribution because N losses from roots and nodules occur during the growing season (Brophy and Heichel, 1989; Ofosu-Budu et al., 1990; Ta et al., 1986). For instance, Rochester et al. (1998) determined that N content in belowground components at R7 represents approximately 60% of N uptake in R5. However, lower NDFA contribution from roots, ranging from 1 to 9%, was recently documented by Gelfand and Robertson (2015). For example, root NDFA contribution could range, on average, from 13 (9% NDFA) to 34 (24% NDFA) kg N ha$^{-1}$ when considering the estimations from Gelfand and Robertson (2015) and Rochester et al. (1998), respectively. As clarified by Anglade et al. (2015), N derived from rhizodeposits are not well contained in a defined physical structure and root N contribution from BNF for all roots, more precisely thinner roots, is very challenging. Variations in root estimates presented in the research literature (Rochester et al., 1998; Gelfand and Robertson, 2015) might as well come from different root sampling techniques, variations in sampling depth, and lack of complete retrieval of in-field N rhizodeposition from thinner roots. It is evident that, after reviewing the scientific literature summarized by Salvagiotti et al. (2008) and considering this current review, more efforts should be focused on collecting data concerning the contribution of roots to obtain a more precise quantification of BNF impact on the partial N balance. In addition, measuring N gains in above- and belowground plant fractions due to BNF are needed, but also, in parallel, monitoring N losses (e.g., including N-metabolites via root excretion) are required.

## SOYBEAN N CREDIT

For soybean N-credit, commonly utilized in US maize–soybean systems for making N-fertilizer recommendations in maize, it could be hypothesized that this N-credit is entirely dependent on soil N mineralization of soybean residues with low C to N ratio (Bundy et al., 1993; Wu et al., 1998; Gentry et al., 2001; 2013). Green and Blackmer (1995) suggested that the N-credit, when sowing corn after soybean as compared with corn as a previous crop, was due to a larger N immobilization in the latter case. In addition, Maloney et al. (1999) and Bergerou et al. (2004) stated that the BNF process plays a minor role in the positive effect of soybean in a maize-soybean rotation, the so-called "soybean rotation effect". An alternative situation could be a transfer of soil N to the following crops, since less soil N is removed when soybean is in the rotation because it uses N derived from BNF, the so-called "N sparing effect" (Chalk, 1998). Even when excluding fertilizer N observations ($n = 190$), the partial N balance still presented an overall −33 kg N ha$^{-1}$ with a very similar data distribution (relative when all fertilizer N points were considered) (50% IQR from −59 to −2 kg N ha$^{-1}$) (Table 3). In any case, it seems that there likely would be a net gain of the partial N balance in the rotation system from BNF, and it may occur at both medium and high BNF groups (Fig. 4B), contributing to the "soybean rotation effect", but there is almost no contribution of the BNF process for the low BNF group (i.e., NDFA below 42%). Seed yield was the main factor driving changes in the partial N balance for the low NDFA group (Fig. 4C), with a more negative N balance as yields increased, showing a decrease of 33.7 kg in the N balance per Mg of yield. However, this relationship became weaker (i.e., low $R^2$) as the NDFA level improved (Fig. 4C). The latter highlights the complexity of N dynamics. A more comprehensive approach looking at losses via greenhouse gases or N leaching (e.g., $N_2O$; Yang and Cai, 2005; Ciampitti et al., 2008; Itakura et al., 2013; Uchida and Akiyama, 2013) and root excretion (Brophy and Heichel, 1989; Ofosu-Budu et al., 1990; Ta et al., 1986), and also N gains or inputs from atmospheric deposition and irrigation water, will improve knowledge and assist in identifying critical components for more precisely estimating the N budget in cropping systems dominated by soybeans.

## OUTCOMES AND FUTURE RESEARCH PRIORITIES

The most noteworthy outcomes of this review are that (i) as the contribution of NDFA increased, seed production per $N_2$ fixation decreased (from 0.033 to 0.017 Mg yield $kg^{-1}$ N from low, 28%, to high, 80%, NDFA; Fig. 1B); (ii) the N-gap increased greatly when NDFA values were above 80% and after a plant N content was above 370 kg N $ha^{-1}$; (iii) when excluding roots, the partial N balance calculation revealed negative values across all the NDFA levels; (iv) the partial N balance was related to $N_2$ fixation, with positive balance most likely to occur for 40% ($n = 41$ points), 15% ($n = 35$), and 3% ($n = 4$) of the database for the high (above 72% $N_2$ NDFA), medium (44 to 72% NDFA), and low BNF (below 44% NDFA) groups, respectively; (v) seed yield was stronger (greater $R^2$) related to negative partial N balance only for the low NDFA group, with no clear trend for the medium and high NDFA groups; and lastly, (vi) the quantity of N contributed from BNF seems negligible, by itself, to be considered as a "soybean N credit" in a maize–soybean cropping rotation, primarily for the low and medium BNF groups. Under this scenario, the apparent N contribution from soybean seems to be primarily related to a greater soil N supply from a more positive N mineralization/immobilization balance in soybean–corn cropping systems.

The observed rise in the N-gap in high-yielding conditions, even with high BNF, suggests the need of having an additional source for supplying N to the crops. This provision has to come primarily from highly efficient *Rhizobium* strains adapted to environments with high plant N demand. However, the development of strategies that supply N at low rates during the cycle (especially during the seed-filling period), reducing the negative impact of soil nitrate concentration on BNF, seems to be a likely solution. These alternatives may involve the inclusion of legume cover crops in the rotation or the use of slow release N fertilizers. Nonetheless, both approaches should evaluate further the impact on soybean productivity, N budget at the crop and system level, and on the environment.

Two research priorities were identified from this review. The first priority should focus on exploring a more holistic study of the N cycling within soybean, first by including a better understanding of the BNF contribution from different plant parts, specifically including roots. Sampling methods and timing within the crop cycle are crucial factors that will affect estimation of root contribution to BNF. The second priority should be to pursue a better understanding of the soil × plant × rhizobia interactions on plant N processes (N remobilization, BNF, and N uptake) in high-yielding soybean systems, especially during the seed filling period, and their contribution to yield and/or seed protein formation process, focusing on the relative importance of contemporaneous and remobilized N. This should be complemented with well calibrated simulation crop models, because it is difficult to have, at the present, field studies that determine N derived from BNF in soybeans that yield more than 7 Mg $ha^{-1}$.

## SUPPLEMENTARY MATERIAL

Supplementary Fig. S1. Relationship between seed yield (adjusted to 13% moisture content) versus $N_2$ fixation (dry basis, kg N $ha^{-1}$) (panel A), and $N_2$ fixation (kg N $ha^{-1}$) versus plant N content (dry basis, kg N $ha^{-1}$) (panel B) for all $N_2$ fixation methods gathered in this review paper.

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